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Screening of the *MEN1* Gene and Discovery of Germ-Line and Somatic Mutations in Apparently Sporadic Parathyroid Tumors

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ABSTRACT

Hyperparathyroidism is the first manifestation in a majority of multiple endocrine neoplasia (MEN1) patients. To discriminate between sporadic and hereditary parathyroid tumors and characterize *MEN1* somatic mutations, we examined *MEN1* gene mutations in patients who had undergone surgery for sporadic parathyroid tumors. DNA was extracted from fresh frozen parathyroid tumor specimens from 112 patients as well as from peripheral blood leukocytes from 64 of the 112 patients. Sequence analysis was performed to examine exons 2–10 of the *MEN1* gene for mutations. Loss of heterozygosity (LOH) was also examined by an analysis of codon 418 and 541, which lie within a polymorphic region of *MEN1*. Somatic *MEN1* mutations were found in 25 of the 112 patients (22%). Two patients had two point mutations (508del33 and Y341X and 363insT and 1767delT, respectively). A total of 27 mutations were characterized, 20 of which have not been reported previously. There were 7 nonsense mutations, 10 frameshift mutations, 2 splice site deletions, 5 missense mutations, and 3 in-frame mutations. Nineteen mutations (70%) predicted truncation of the menin protein. Germ-line *MEN1* mutations were found in 3 of 64 patients (5%) who had no family history of endocrine tumors associated with MEN1, and these patients were identified as *MEN1* gene probands. LOH at the *MEN1* locus was detected in three parathyroid tumors showing germ-line mutation. LOH was significantly frequent in parathyroid tumors with somatic *MEN1* mutations (15 of 22 tumors, 68%) but not in those without germ-line or somatic *MEN1* mutations (14 of 51 tumors, 28%; $P = 0.0011$). Our findings suggest that alterations of both alleles of the *MEN1* gene may be associated not only with endocrine tumors of affected MEN1 patients but also with sporadic parathyroid tumors. Germ-line *MEN1* gene analysis can distinguish heritable from nonheritable parathyroid tumors, and *MEN1* gene evaluation of patients with apparently sporadic parathyroid tumor is recommended before parathyroid surgery.

INTRODUCTION

Parathyroid adenoma and hyperplasia are commonly found and are the most frequent causes of primary hyperparathyroidism. Hypercalcemia can cause nephro-urolithiasis, osteoporosis, pancreatitis, and psychiatric disorders. The sporadic form is very common; the hereditary form is also well known. MEN1[2] is an inherited cancer syndrome characterized by three endocrine tumors in different combinations: (*a*) parathyroid hyperplasia; (*b*) pancreatico-gastrointestinal neuroendocrine tumor; and (*c*) pituitary tumor. Segregation is autosomal dominant, and the overall incidence of hyperparathyroidism is more than 90% in MEN1 patients (1, 2). The prevalence of pancreatico-gastrointestinal tumors and pituitary tumors is 40–70% and 30–60%, respectively. In 1997, germ-line mutations of the *MEN1* gene were identified and have been found in members of families afflicted with MEN1 (3, 4). The *MEN1* gene consists of 10 exons, and it encodes a putative 610-amino acid (M_r 67,000) nuclear/cytoplasmic polypeptide, menin, with two nuclear localization signals (5–8). *MEN1* germline mutations have been found throughout the coding exons of the *MEN1* gene, and no mutational hot spots have been found (9, 10). No genotype-phenotype correlation has been elucidated in MEN1 patients. In the clinical management of MEN1 families, direct molecular analysis of *MEN1* gene mutations is replacing conventional genotyping and biochemical screening to discriminate between gene carriers and non-gene carriers.

In sporadic parathyroid tumors, frequent LOH on chromosome 11q13, the chromosome on which the *MEN1* gene lies, has also been found (11). Of a small number of parathyroid tumors investigated recently, 15–21% had somatic mutations in the *MEN1* gene (12–14). No germ-line mutation was found in these studies. We investigated *MEN1* gene mutations in a larger number of patients with apparently sporadic parathyroid tumors and found both germ-line and somatic mutations.

MATERIALS AND METHODS

A total of 112 patients for whom the preoperative diagnosis was sporadic parathyroid tumor underwent parathyroidectomy at Noguchi Thyroid Clinic and Hospital Foundation between 1989 and 1998. The average age of patients was 57.8 ± 13.1 years; 14 patients were men, and 98 were women. Uniglandular disease was diagnosed intraoperatively in 104 patients, and 8 patients were diagnosed with multiglandular disease. The median tumor weight was 758 mg (quartile points, 326 and 1660 mg) in the patients with uniglandular disease and 231.1 mg (quartile points, 52.7 and 624.6 mg) in the patients with multiglandular disease. The histopathological diagnosis, based on parathyroid gland specimens, was parathyroid adenoma in 67 patients, hyperplasia in 44 patients, and adenolipoma in 1 patient. There was no apparent family history of MEN1, hereditary parathyroid tumor, pancreatico-gastrointestinal endocrine tumor, and pituitary tumor at the time of initial evaluation in any patient. A family history of cancer was present in 34 patients, including 2 patients with a family history of pancreatic cancer. No other disease, including tumor of the pancreas or duodenum or pituitary tumor coupled with hyperparathyroidism, was detected in these patients. A history of thyroid disease was present in seven patients, a history of thyroid cancer was present in one patient, a history of nodular goiter was present in one patient, and a history of Graves' disease was present in five patients. Forty-four patients showed thyroid disease and underwent parathyroidectomy and thyroidectomy. The histopathological diagnosis, based on thyroid gland tissue, was thyroid cancer in 16 patients, adenomatous goiter in 13 patients, follicular adenoma in 12 patients, Graves' disease in 1 patient, chronic thyroiditis in 1 patient, and malignant lymphoma of the thyroid in 1 patient.

DNA was extracted from fresh frozen parathyroid tumors from 112 patients, and PBLs were extracted from 64 patients as described previously (15). All patients subjected to somatic and/or germ-line analysis gave informed consent before participation in the MEN1 study. Oligonucleotide primers for exons 2–10 of the *MEN1* gene were synthesized as described by Lemmens *et al.* (4) The PCR amplification reaction was carried out in a 50-μl mixture containing 100 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 5–10 pmol of each sense and antisense primer, and 1 unit of Ampli Taq Gold (Perkin-Elmer Biosystems, Foster City, CA) with a PROGENE programmable thermal cycler (Techne, Cambridge, United Kingdom). After initial

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[2] The abbreviations used are: MEN1, multiple endocrine neoplasia type 1; LOH, loss of heterozygosity; PBL, peripheral blood leukocyte.

denaturation at 94°C for 12 min, PCR was carried out for 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C to 67°C, and a polymerase reaction for 1 min at 72°C, followed by a 7-min final extension at 72°C.

For nonisotopic cycle sequencing, DNA products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). These purified products were subjected to an additional 25 PCR cycles with sense or antisense primer by fluorescence-based dideoxy terminator cycle sequencing (Perkin-Elmer Biosystems). These products were then eluted through a centri-sep spin column (Perkin-Elmer Biosystems) and subjected to capillary gel electrophoresis. Data collection and analysis were performed on an automated DNA sequencer (Model 310; Perkin-Elmer Biosystems). All PCR reactions and sequencing were performed repeatedly, and we confirmed the presence or absence of *MEN1* mutation.

LOH was investigated at polymorphic sites located at codons 418 (GAC/GAT) and 541 (GCA/ACA). The nonneoplastic counterpart of DNA was available by PBLs from 64 patients. When either exon was heterozygous, the case was judged as informative. In informative cases, when loss of one heterozygous nucleotide was seen in the parathyroid tissue, the tumor was judged as having LOH.

RESULTS

Table 1 summarizes the *MEN1* mutation in apparently sporadic parathyroid tumors. Mutations were found in 25 of 112 patients (22%). Two simultaneous mutations were found in two patients: (*a*) 508del33 and Y341X in patient 21; and (*b*) 363insT and 1767delT in patient 28. A total of 27 mutations was found; 20 of these mutations had not been reported previously, with the exceptions being R98X, R108X, G156D, W183R, Q209X, Y341X, and 1657insC. Mutations were distributed throughout exons 2–10, intron 2, and intron 6 of the *MEN1* gene and were seen most frequently in exon 2 (Fig. 1). Seven of the mutations were nonsense mutations, 10 were frameshift mutations, 2 were splice site deletions, 5 were missense mutations, and 3 were in-frame mutations. Nineteen mutations (70%) predicted truncation of the menin protein, and 8 (30%) encoded amino acid substitutions without truncation of the menin protein.

The presence or absence of *MEN1* mutations in parathyroid tumors was not associated with any clinicopathological parameter such as age, sex, tumor site, uni- or multiglandular disease, weight of resected parathyroid gland, or histological diagnosis (Table 2).

We examined germ-line *MEN1* mutation by using PBLs from 64 patients. PBLs were available from 17 of 25 patients with *MEN1* mutation in parathyroid tissue (Table 2). In patients with S253W, E274A, and 1668insT mutations, the mutations were found in both parathyroid tissue and PBLs, and these patients (patients 102, 39, and 43) were judged as having germ-line mutations (3 of 64 patients, 5%). Patient 102, a 30-year-old man, was diagnosed as having single glandular disease preoperatively and intraoperatively. Left lower parathyroidectomy was performed, and the resected parathyroid gland was 500 mg. The histological diagnosis was parathyroid hyperplasia, and hypercalcemia in this patient has persisted postsurgery. A S253W germ-line point mutation was found in this patient. Patient 39, a 25-year-old man, was diagnosed as having single glandular disease preoperatively and intraoperatively. Upper right parathyroidectomy was performed, and the resected parathyroid gland was 180 mg. The histological diagnosis was parathyroid hyperplasia. To date, 7 years after surgery, the serum calcium level is mildly high, and hypophosphatemia is present. This patient demonstrated an E274A germ-line point mutation. Patient 43, a 56-year-old woman, was diagnosed as having single glandular disease preoperatively and intraoperatively. Left lower parathyroidectomy was performed, and the resected parathyroid gland was 5490 mg. The histological diagnosis was parathyroid hyperplasia. Persistent hypercalcemia is present. Six years after surgery, hyperparathyroidism was diagnosed in her sister, and pancreatic gastrinoma was diagnosed in her cousin. A 1668insT germ-line mutation was found in this patient.

We determined the LOH at the genetic locus spanning exon 9 containing codon 418 and exon 10 containing codon 541. At codon 418, the allele frequency was 66% as GAC and 34% as GAT ($n = 134$). Heterozygosity at codon 418 was found in 31 of 67 (46%) patients. Allele frequency at codon 541 was 75% GCA and 25% ACA

Table 1 *MEN1 gene mutation in apparently sporadic parathyroid tumors*

Patient no.	Sex	Age (yrs)	Parathyroid weight (mg)	Histology[a]	Exon	Codon	Mutation	Type of mutation[b]	Germ-line[c]	LOH[d]
93	F	59	130	H	2	3	118 del35/insGGCCT	I	w-t	−
108	F	50	3930	A	2	54	272 insG	F	w-t	−
113	F	49	410	A	2	55	273 del11	F	w-t	LOH
131	F	73	840	A	2	67	309 delCCCG	F	−	LOH
28	F	70	2530	H	2	85	363 insT	F	−	−
91	F	75	480	A	2	97	401 delCC	F	w-t	LOH
75	F	64	1310	A	2	98	R98X	N	−	LOH
133[e]	F	75	230	H	2	108	R108X	N	w-t	LOH
21	F	71	130	A	2	133	508 del33	I	−	−
56	F	53	350	H		Intron 2	555 +2del ttgg	S	−	−
73	F	74	100	A	3	156	G156D	M	w-t	LOH
58	F	43	5170	A	3	209	Q209X	N	−	LOH
120	F	71	220	H	3	183	W183R	M	w-t	−
102	M	30	500	H	4	253	S253W	M	Mutant	LOH
39	M	25	180	H	5	274	E274A	M	Mutant	LOH
29	F	49	1710	A	6	284	A284P	M	w-t	LOH
54	F	50	980	H		Intron 6	1022 + 1del g	S	w-t	LOH
49	F	63	1160	A	7	310	K310X	N	w-t	LOH
21	F	71	130	A	7	341	Y341X	N	−	−
64	F	60	660	A	8	358	E358X	N	w-t	LOH
40	F	45	1710	A	8	387	1265 insG	F	w-t	LOH
126	F	59	420	H	9	414	1350 del36	I	−	LOH
53	F	34	1370	A	10	454	1470 delA	F	w-t	−
48	F	68	550	A	10	516	1657 insC	F	−	LOH
43	F	56	5490	H	10	520	1668 insT	F	Mutant	LOH
118	F	60	610	H	10	543	S543X	N	w-t	LOH
28	F	70	2530	H	10	553	1767 delT	F	−	−

[a] H, hyperplasia; A, adenoma.
[b] M, missense mutation; I, in-frame mutation; F, frameshift mutation; N, nonsense mutation; S, splice site deletion.
[c] w-t, wild-type (sporadic type); Mutant, mutant-type (hereditary type); −, PBL was not available.
[d] LOH status in parathyroid tumor; −, retain heterozigosity.
[e] Patient 133 had multiglandular disease. All patients listed in Table 1 except for patient 133 had uniglandular disease.

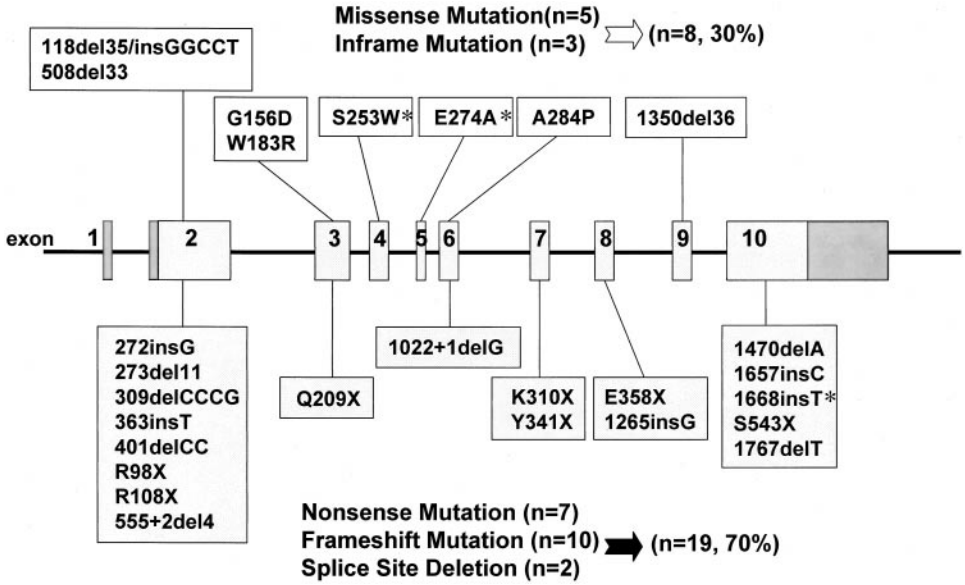

Fig. 1. Spectrum of mutations in the *MEN1* gene in parathyroid tumors. *Numbered boxes* are exons 1–10 of the *MEN1* gene; the coding region is exons 2–10, and introns are shown by a *thick horizontal line* (not drawn to scale). Twenty-seven mutations were found; mutations resulting in truncation of menin protein are shown *below* the line, and others are shown *above* the line. *Asterisks* indicate germ-line mutations.

($n = 132$). Heterozygosity at codon 541 was found in 19 of 66 (29%) patients. Combining the results of codons 418 and 541, 72 of 112 (64%) patients were informative, and 40 of 112 (36%) patients were not informative in this study. In addition, LOH was judged by the data from mutated locus of two patients with *MEN1* mutation in parathyroid tissue. In the present series, LOH was found in 30 of 74 (41%) informative cases, and 16 of 30 (53%) LOH-positive parathyroid tumors had somatic *MEN1* mutations. LOH in parathyroid tumors was found in three of three (100%) informative cases with germ-line *MEN1* mutations. Significant LOH was found in informative parathyroid tumors with somatic *MEN1* mutations (15 of 22 tumors, 68%) as compared with LOH found in informative tumors without *MEN1* mutations (14 of 51 tumors, 28%; $P = 0.0011$).

DISCUSSION

This study demonstrates the involvement of *MEN1* gene mutations in patients with sporadic parathyroid tumors and reveals germ-line mutation in apparently sporadic parathyroid tumors. Earlier studies have shown *MEN1* mutations in some sporadic parathyroid tumors. Heppner *et al.* (12) reported *MEN1* mutations in 7 of 33 (21%) parathyroid tumors, and these tumors also showed LOH. Farnebo *et al.* (13) reported *MEN1* mutations in 6 of 40 (15%) parathyroid tumors. They found that tumors showing LOH and mutation were significantly larger than tumors without LOH and mutation. Carling *et al.* (14) reported *MEN1* mutation in 6 of 13 parathyroid tumors with LOH at 11q13 but reported no significant differences in clinical indices.

In other sporadic endocrine tumors, somatic *MEN1* mutation was found in 9 of 27 (33%) gastrinomas and in 2 of 12 (17%) insulinomas (16). In contrast, pituitary tumors had infrequent *MEN1* mutations (17–19). No somatic *MEN1* mutation was found in adenomas, hyperplasias, and carcinomas of adrenocortical lesions, and germ-line mutation was reported in only one patient with apparently sporadic adrenocortical adenoma (20). In sporadic carcinoid tumors of the lung, 4 of 11 (36%) patients showed somatic *MEN1* mutation (21). Although *MEN1* gene inactivation may have an important role in the development of endocrine tumors of various organs in patients with MEN1, the difference in the incidence of somatic *MEN1* mutation in tumors suggests the involvement of other genes in sporadic endocrine tumor development.

In the present study, no association was found between the somatic *MEN1* mutation and clinicopathological parameters in parathyroid tumors. Loss of function of menin protein in sporadic parathyroid tumors is not always associated with multifocality or proliferative activity of parathyroid disease. Although only one patient with multiglandular disease showed *MEN1* mutation, three patients initially

Table 2 *Clinicopathological parameters of MEN1 mutation-positive and negative parathyroid tumors*

Parameters	*MEN1* mutation		*P*
	Positive (%)	Negative (%)	
Age (yrs)	57.2 ± 13.9	57.9 ± 12.9	0.820[a]
Sex			
Male	2 (14.3)	12 (85.7)	0.732[b]
Female	23 (23.5)	75 (76.5)	
Disease			
Uniglandular	24 (23.1)	80 (76.9)	0.681[b]
Multiglandular	1 (12.5)	7 (87.5)	
Resected parathyroid gland weight (median)	609.5 (292.4, 1537.4)[c]	753.1 (304.0, 1636.2)[c]	0.553[a]
Histological diagnosis			
Adenoma	15 (22.4)	52 (77.6)	0.926[d]
Hyperplasia	10 (22.7)	34 (77.3)	
Adenolipoma	0 (0)	1 (100)	

[a] Statistical significance was calculated by *t* test.
[b] Statistical significance was calculated by Fisher's exact test.
[c] (Lower quartile point, higher quartile point).
[d] Statistical significance was calculated by Pearson's χ^2 test.

diagnosed as having uniglandular disease were determined to have germ-line *MEN1* mutations and were inadvertently included in the uniglandular group. In the absence of any genetic testing, the surgeon determines which parathyroid gland of a patient with hyperparathyroidism is affected either preoperatively or intraoperatively. In MEN1 patients, multiglandular disease occurs. However, there is a wide heterogeneity in size of the parathyroid glands in MEN1 patients and in patients with sporadic primary hyperparathyroidism (22). It is impossible to discriminate perfectly between MEN1 and non-MEN1 patients by clinical features, results of clinical examinations, and macroscopic view of parathyroid glands (23). Multiglandular disease can be missed in centers that do not make a strong effort to identify two and preferably four glands at all initial operations. Genetic testing of the germ-line *MEN1* gene or other parathyroid-related genes may predict before surgery whether the parathyroid process is multiglandular or uniglandular.

By combining the results of tumor tissue and PBLs in codons 418 and 541, it was possible to obtain 65% informative cases at the *MEN1* allele. Codon 541 is a highly polymorphic site among the Japanese, and there is an ethnic difference associated with the allele frequency of this codon (9). LOH was found in 68% of parathyroid tumors showing somatic *MEN1* mutation. If two mutations in two individuals (patients 21 and 28) occurred independently in paternal and maternal chromosomes, these individuals also had genetic alterations on both alleles of the *MEN1* gene, and 17 of 22 (77%) would have inactivation of both alleles of the *MEN1* gene. In the remaining five tumors with somatic mutation but without LOH at the *MEN1* locus, nonneoplastic cells may be intermingled with tumor cells. In contrast, in parathyroid tumors without *MEN1* mutation, only 28% showed LOH at the *MEN1* locus. These results suggest that the *MEN1* gene may operate as a tumor suppressor gene, and loss of function of the menin protein may have an important role in the development of parathyroid tumors. In our series, 53% of parathyroid tumors with LOH at the *MEN1* allele showed somatic *MEN1* mutation. A similar relationship between the frequency of LOH and mutations has been reported previously (12–14). In mutation-negative, LOH-positive tumors, mutations in the noncoding regions of the *MEN1* gene, inactivation of the *MEN1* gene by methylation, or inactivation of other unknown tumor suppressor gene(s) existing near the *MEN1* locus may be present and may play a role in the development of parathyroid tumors (24).

Germ-line *MEN1* mutations were found in 3 of 64 (5%) patients with apparently sporadic parathyroid tumors in this study. These three patients showed only one recognized parathyroid gland and underwent a single gland resection. These patients have persistent or recurrent hyperparathyroidism. These patients were genetically diagnosed as MEN1 probands. Examination of the pancreas, pituitary gland, or adrenal gland is necessary. Furthermore, a family study discriminating gene carriers from non-gene carriers is possible. However, after we informed these patients of the results of the genetic diagnosis, they developed serious psychological problems, anxiety about their future, or a distrust of the first operation. This made it difficult to persuade them to undergo reexamination, to undergo subsequent operations for hyperparathyroidism, to undergo exploration to examine the pancreas and pituitary gland, or to have their genetic family screening analyzed. Elucidating germ-line *MEN1* mutation before the initial parathyroid surgery is most important in these patients. In Japan, the fraction of MEN1 kindreds among all MEN1 probands was small in contrast to the larger fraction seen in Europe and in the United States (1, 10, 25–27). The frequency of undiscovered MEN1 germ-line mutation may differ in each country because this is partly a social issue in certain countries.

A literature review concluded that MEN1 (it was not stated whether this referred to known or occult MEN1) could account for 2–3% among all hyperparathyroidism (28). Muhr *et al.* (29) reported that clinical examination and hormonal evaluation of 63 patients with hyperparathyroidism did not reveal any signs of endocrine disease suggestive of MEN1. Hyperparathyroidism is the first manifestation of disease in a majority of MEN1 patients, and the age at onset of parathyroid tumor in patients with MEN1 was about 20 years earlier than the age at onset of sporadic parathyroid tumor (1, 2, 30). Bassett *et al.* (25) calculated age-related penetrances of MEN1. According to their calculations, the age-related penetrance of MEN1 is 52%, 87%, and 98% at 20, 30, and 40 years of age, respectively. To find a new MEN1 family, examination of *MEN1* gene mutation before the initial treatment of hyperparathyroidism may be the most effective approach.

The total number of nucleotides of the *MEN1* gene we must examine, including exon-intron boundaries, is about 2 kb. Moreover, we must divide the *MEN1* gene into 10–12 PCR fragments to perform the analysis. At least 1–2 weeks are needed to confirm the presence or absence of *MEN1* mutation for each patient. Thus, the present technique for discovering new MEN1 families is time-consuming and expensive. Furthermore, *MEN1* mutation cannot be found in 10–15% of MEN1 families. Although these technical and scientific problems are present, preoperative genetic screening of the germ-line *MEN1* gene in patients with apparently sporadic parathyroid tumors will be a useful method for discriminating between hereditary and sporadic parathyroid tumors.

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